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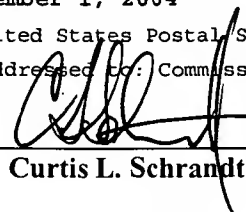
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PROVISIONAL APPLICATION COVER SHEET

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MOLECULAR CHARACTERIZATION OF A NOVEL PATTERN RECOGNITION PROTEIN FROM NONSPECIFIC CYTOTOXIC CELLS: SEQUENCE ANALYSIS, PHYLOGENETIC COMPARISONS AND ANTIMICROBIAL ACTIVITY OF A RECOMBINANT HOMOLOGUE

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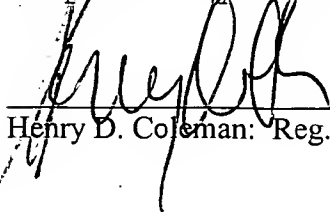
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MOLECULAR CHARACTERIZATION OF A NOVEL PATTERN RECOGNITION
PROTEIN FROM NONSPECIFIC CYTOTOXIC CELLS: SEQUENCE ANALYSIS,
PHYLOGENETIC COMPARISONS AND ANTIMICROBIAL ACTIVITY OF A
RECOMBINANT HOMOLOGUE.

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Abstract

Nonspecific cytotoxic cells (NCC) are the first identified and most extensively studied killer cell population in teleosts. NCC kill a wide variety of target cells including tumor cells, virally transformed cells and protozoan parasites. The present study identified a novel evolutionarily conserved oligodeoxynucleotide (ODN) binding membrane protein expressed by channel catfish (*Ictalurus punctatus*) NCC. Peptide fingerprinting analysis of the ODN binding protein (referred to as NCC cationic antimicrobial protein-1/ncamp-1) identified a peptide that was used to design degenerate primers. A catfish NCC cDNA library was used as template with these primers and the PCR-amplified product was sequenced. The translated sequence contained 203 amino acids (molecular mass of 22,064.63 Daltons) with characteristic lysine rich regions and a pI = pH 10.75. Sequence comparisons of this protein indicated similarity to zebrafish (51.2%) histone family member 1-X and (to a lesser extent) to trout H1. A search of EST databases confirmed that ncamp-1 is also expressed in various tissues of channel catfish as well as zebrafish. Inspection for signature repeats in ncamp-1 and comparisons with histone-like peptides from different species indicated the presence of multiple lysine based motifs composed of AKKA or PKK repeats. The novel protein was cloned, expressed in *E. coli* and used to generate rabbit antiserum. The recombinant ncamp-1 bound GpC and CpG ODNs and was detected with homologous anti-ncamp-1 polyclonal antibodies. Western blots of NCC membranes using anti-ncamp-1 serum detected a 29 kDa protein. Binding competition experiments demonstrated that anti-ncamp-1 antibodies and GpC bound to the same protein on NCC. Two different truncated forms of ncamp-1 as well as the full-length recombinant protein exhibited antimicrobial activity. The present study demonstrated the expression by NCC of a

new membrane protein that may participate in the recognition of bacterial DNA and as such participate in innate antimicrobial immune responses in teleosts.

Key Words. Nonspecific cytotoxic cells (NCC); antimicrobial proteins, oligodeoxynucleotides, lysine box motif, innate immunity, pattern recognition receptors, natural killer cells.

INTRODUCTION

Bacterial DNA initiates inflammatory responses and is responsible for development of some level of innate immunity in mammals [1-4]. These actions are principally attributed to the presence of relatively high concentrations (compared to eukaryotic DNA) of unmethylated CpG dinucleotide motifs. Studies have shown a wide diversity of functional oligodeoxynucleotide (ODN) sequences. Dinucleotide versus single base composition [5]; different structural configurations (G-tetrads/guanosine quadruplexes) [6-7]; as well as the most effective “backbone” (hexose-phosphate) derivatizations (phosphodiester versus phosphorothioate) [8] have all been reported to influence effector cell activity in different animal species.

There are several different classes/types of DNA binding proteins on mammalian cells represented by a limited number of germ-line encoded receptors that are expressed predominantly on antigen presenting cells (with low levels of expression on T-cells and NK cells). These proteins are referred to as pattern recognition receptors (PRR) [9-10] and they bind to pathogen associated molecular pattern ligands (PAMP) of microbial origin. The PAMPs include LPS, peptidoglycan, certain lipoproteins, CpG oligodeoxynucleotides (ODNs), etc. The most widely distributed of the PRR that bind ODNs are the Toll-like receptor 9, Mac-1 (CD 11b/CD18) [11-15] and Scavenger receptor-A [16-17]. ODN binding to PRR may cause either activation or inhibitory responses depending on the ODN concentration and target cell type. In addition, ligation of PRR *in vivo* by ODNs may also produce different pathways of immunoregulation such as autoimmunity, Th1 bias activation, etc. [18-20].

Studies of anti-bacterial innate cellular responses in teleosts provides an interesting evolutionary/phylogenetic model for mechanisms of defense. In higher vertebrates the (direct) binding and functional activation of NK cells by bacterial DNA has not been defined and the

expression of DNA binding proteins on these cells in many cases remains controversial.

Nonspecific cytotoxic cells (NCC) directly bind to and are activated by ODNs [21]. In the present study an ODN binding histone-like protein from NCC was identified, sequenced and a recombinant homologue was expressed.

Histone proteins have been previously detected in membrane preparations from human leukocytes [22-23]; monocytes [24]; a T-cell line (HPB-ALL) [25]; and an H1-like protein has been described from neuronal cells [26]. A 30 kDa DNA receptor was identified [27-28] on the membrane of human leukocytes. Evidence for the association of cell-derived and/or cell membrane histone H1 as a participant in antibacterial innate immunity has also been provided by studies of human ileal mucosal extracts [29] and human ulcerative colitis (UC) [30]. In both cases H1 was either released from villus epithelial cells [29] or was associated with a serum marker for UC [30]. In addition, Raji cells express 14, 17, 18, 33 and 34 kDa DNA binding proteins [31]. These histone or histone-like proteins were described as being responsible for the binding, endocytosis and degradation of exogenous DNA. Interestingly, the thyroglobulin receptor on the cell surface of J774 (mouse) macrophages is an H1 protein [32].

Teleosts appear to be the most abundant source [33-38] for model studies of the role of histone-like proteins in antimicrobial immunity. These studies have demonstrated that histones and histone-like proteins exist in a wide variety of teleost tissues and they exhibit antimicrobial and antiparasitic activities. Here we demonstrate the expression of a novel cationic ODN binding protein (ncamp-1) on NCC membranes. The expressed recombinant ncamp-1: bound to ODN; was used to produce polyclonal antiserum; and had anti-bacterial activity. The membrane expression of ncamp-1 was confirmed by flow cytometry and by Western blots of NCC membrane lysates using the anti-ncamp-1 antiserum. Finally, the recombinant ncamp-1 killed

Gram positive and Gram negative bacteria. We postulate that ncamp-1 may comprise a new class of pattern recognition proteins involved in recognition of bacterial DNA and amplification of teleost innate immunity.

Materials and Methods

Media, reagents and antibodies. Cells were cultured in RPMI-1640 (Cellgro, Media Tech, Washington, DC) supplemented with L-glutamine, sodium pyruvate, MEM vitamin solution, MEM amino acid solution, MEM non-essential solution (Cellgro), 50 mg/ml gentamicin (Schering-Plough Animal health Corp., Kenilworth, NJ) and 10% fetal bovine serum (FBS) (Atlanta biologicals, Norcross, GA). PAB solution contained phosphate buffered saline with 0.1% sodium azide and 1% bovine serum albumin. Calf thymus DNA (#D-4764) and Extravidin-phycoerythrin (PE) conjugate (# E4011) were purchased from Sigma. Cells in all assays were 70-90% mab 5C6 positive. Monoclonal antibody 5C6 (IgM isotype) specific for a 32-kDa receptor protein (i.e. NCCRP-1; 39) was prepared in-house. Anti-mouse IgM-FITC and biotin-anti-IgM conjugates were obtained from Sigma Immunochemicals. Rabbit polyclonal anti-recombinant ncamp-1 was generated by the Polyclonal Antibody Production Service at the University of Georgia using standard immunization protocols with His-Tag purified Ncamp-1 protein (see below) as immunogen. The IgG fraction was isolated by Protein A agarose (IPA-300, Repligen, Cambridge MA) chromatography. Normal rabbit serum (NRS; E-9133, Sigma) was treated similarly for use as negative control IgG.

Oligodeoxynucleotides (ODNs). ODNs were purchased from MWG-Biotech (High Point, NC). All ODNs were synthesized as a phosphodiester. Three prime end modifications (biotin or rhodamine) were done by the manufacturer. The ODNs were: TCGTCGTTGTCGTTGTCGTT (CpG); TGCTGCTTGTGCTTGTGCTT (GpC); 20 mers of

polyguanosine (dG20); and 20 mers of polyadenine (dA20). ODNs were resuspended in PBS prepared in endotoxin free water (#210-7, Sigma). All ODNs contained less than 0.015 EU/ml endotoxin as determined by the Limulus ameobocyte lysate assay (E-Toxate Sigma).

Experimental animals and isolation of NCC. Channel catfish weighing 20-60g were net captured and sacrificed by submersion in anesthetic (3-aminobenzoic acid ethyl ester; #D-5040 Sigma). Anterior kidney (AK) tissue (mammalian bone marrow equivalent) was removed aseptically and passed through screen mesh to obtain single cell suspensions in complete RPMI-1640 containing 10% FBS. Red cells were first removed by one cycle of centrifugation through Ficoll-Hypaque, (400 x g/30 min/room temp). Cells were harvested and purified centrifugation over a 45.5 % Percoll cushion. Cells at the interface were collected, washed once with RPMI and resuspended as indicated.

Flow cytometry. The ability of NCC to bind was ODNs was assessed by flow cytometric analysis. Rhodamine-labelled CpG was added to purified NCC cells resuspended in PAB. Cells were incubated on ice for 45 min washed with PAB and analyzed. The surface expression on NCC of ncamp-1 was evaluated using polyclonal rabbit IgG prepared against recombinant ncamp-1. Purified NCC in PAB was incubated with antibody or control rabbit IgG for 1 hour on ice. Cells were washed in PAB, anti-rabbit IgG FITC was added (1 hour on ice). Cells were washed in PAB and analyzed. Analysis was performed using an EPICS XL-MCL four color analyzer (Coulter Electronics Corp, Hialeah, FL), equipped with 15 mW air cooled argon-ion laser operating at 488 nm wavelength. FITC was detected using 525nm bandpass filter by photomultiplier tube 1 (PMT1) and the rhodamine and PE signals were detected with 575nm bandpass filter by photomultiplier tube 2 (PMT2). Data was analyzed using Coulter's System II software, version 3.0.

Competitive binding. Catfish NCC were purified as described previously except were subjected to two cycles of Ficol-Histopaque centrifugation prior to centrifugation on 45.5% Percoll. Purified cells were resuspended in PAB. Labeled ODNs were used as previously described (21). Polyclonal anti-ncamp-1 IgG (or NRS IgG) or biotinylated ODN was added to cells and incubated for 45 minutes on ice. Cells were washed in PAB and the appropriate second reagent was added (i.e. antibody was added to cells first followed by addition of labeled ODN; or labeled ODN was added to cells first followed by the addition of antibody). Samples were incubated for 45 min on ice, washed in PAB and further incubated (45 min on ice) in either Extravidin-PE or anti-rabbit IgG FITC as indicated.

Preparation of cell membranes. Purified NCC were washed three times with ice cold TBS (25mM Tris-Cl, pH 7.5, 150 mM NaCl). Cells were resuspended in Dounce homogenization buffer (10mM Tris-Cl, pH 7.6, 0.5mM MgCl₂, 1 µg/ml each leupeptin, pepstatin and aprotinin; 1mM PMSF) at 2×10^7 cells/ml and incubated on ice for 15 min prior to homogenization with 50-60 strokes of a Dounce homogenizer (type B pestle). Salt was adjusted to 0.15 M by the addition of tonicity restoration buffer (10mM Tris-cl, pH 7.6, 0.5mM MgCl₂ and 0.6M NaCl) per ml of homogenization buffer and cells were spun at 500g for 5 min to remove nuclei. Supernatant was collected and EDTA was added to 5mM. Supernatant was then spun at 13000 rpm for 10 min, 4°C. Supernatant was discarded and the pellet was washed twice with cold TBS with protease inhibitors (as above). Membrane pellets were resuspended in hot SDS PAGE sample buffer at 2×10^8 cell equivalents (CE)/ml.

Southwestern (ODN) blots. Biotinylated ODNs were used to identify potential DNA binding proteins in NCC membrane lysate preparations and to evaluate the ODN binding of recombinant ncamp-1. Membrane lysates were prepared as indicated above. Purified

recombinant ncamp-1 was mixed with SDS-PAGE sample buffer (reducing) prior to electrophoresis. Samples were run in 12.5% SDS-PAGE and transferred to nitrocellulose. Membranes were blocked in Superblock (Pierce, Rockford IL) blocking solution with 0.05% Tween -20. ODN's were diluted in Superblock and added to membranes (1 h, room temperature). Membranes were washed (3X) in TBS with 0.5% Tween-20 (TBST). Neutravidin-HRP (Pierce) diluted in Superblock (minimum dilution 1:50000) was added and incubated for 30 min at room temperature. Blots were washed (3x) in TBST and were developed with enhanced chemiluminiscence (SuperSignal® West Pico Pierce, Rockford, IL).

Western blot. Anterior kidney NCC membrane preparations were resolved on 12.5% gels and transferred to nitrocellulose for Western blot analysis. Filters was blocked for 30 minutes at room temperature (RT) with blocking buffer (5% non-fat dry milk in TBST), incubated with rabbit anti-ncamp-1 IgG (or control IgG) followed by washing and anti-rabbit IgG-Horseradish Peroxidase conjugate diluted 1:10000 in blocking buffer. Blots were developed with enhanced chemiluminescence as above.

Protein fingerprinting, primer design and PCR amplification. Proteins identified by ODN binding in Southwestern blots were excised from Coomassie stained gels and protein fingerprinting was done by microcapillary reverse phase HPLC followed by ion trap mass spectrometry (MS) (Harvard Microchemistry facility). The MS spectra of peptide fragments were compared (using an algorithm called Sequest) and the results were manually verified by checking the fidelity of the run and biological significance. One of the peptide fragments identified had high degree of similarity to the MS spectra of a peptide fragment from histone H1 from trout. This peptide fragment was used to design degenerate primers to amplify portions of the gene (in combination with vector specific primers for the library) using cDNA library

constructed from NCC purified from catfish anterior kidney as a template. The amplicons were cloned in to a pDrive TA cloning vector (Qiagen, Carlsbad, CA) and sequenced in a 373 A DNA sequencer (Applied Biosystems, Foster City, CA) at the Molecular Genetics Instrumentation Facility (University of Georgia, Athens) using the standard protocol described by the manufacturer. Sequences were compared with the known sequences in DDBJ/EMBL/GenBank databases using BLAST version 2.2.5. Based on the sequence, which had similarity to H1 histone family X members, non-degenerate primers were designed to screen the cDNA library using a directed PCR-based iterative cloning protocol. Several clones were sequenced in both directions to verify the complete sequence.

Recombinant protein. Primers were designed to amplify the entire coding region of ncamp-1 to generate the recombinant protein. PCR amplified and restriction digested insert DNA was directionally cloned in to pET-21 b expression plasmid (Novagen, San Diego, CA), which allow the expression of protein with C-terminal His-Tag. The resulting plasmid (pET-21b-ncamp) was electroporated into *E. coli* expression strain BL21(DE3)pLysE (Novagen). Recombinant protein was produced from bulk cultures grown till 0.6 – 1.0 OD and induced with 1mM isopropyl-beta-D-thiogalactopyranoside (IPTG, # BP1620-1, Fisher, Fair Lawn, NJ) for 3 h at 30°C. Lysates were prepared from IPTG induced cultures by sequential incubations in lysozyme (1mg/ml), Triton X-100 (0.5%), DNaseI (5 µg/ml) and RNase A (10 µg/ml). Ncamp-1 was purified from cleared lysates using Ni-NTA-agarose (#30210, Qiagen, Valencia, CA) according to manufacturer's instructions. Mock (vector) lysates were by generated similarly from bacteria transformed with pET21b vector only and were “purified” on NiNTA beads for use as negative controls where indicated.

Anti-microbial assay with recombinant ncamp-1. The recombinant protein was examined for anti-microbial activity by a modified broth dilution assay. Ncamp-1 activity was tested against Gram positive (*Micrococcus luteus*, *Streptococcus iniae*) and Gram negative (*E. coli*, DH5alpha strain) microorganisms. *S. iniae* was grown and tested for susceptibility in Todd-Hewitt broth (THB) at 30C. *E. coli* and *M. luteus* were grown and tested at 37C in Mueller-Hinton broth (MHB). Overnight cultures were diluted into fresh media and grown at the indicated temperature for 3-6 h. Cultures were diluted to 5000 CFUs (colony forming units)/ml in 10 mM sodium phosphate buffer (NAPB), supplemented to 10% with either MHB or THB. The relationship between A₆₂₀ and CFUs was previously determined in our laboratory for each bacteria. Fifty microliters of bacteria (5000 CFUs) were added to an equal volume of ncamp-1 (or control protein) diluted in 10 mM NAPB to the indicated protein concentration. Assay tubes were incubated at the appropriate temperatures for 2 h with shaking. Colony counts were then determined by plating serial dilutions of test cultures on the appropriate media. Results are expressed as CFUs/ml.

Results

Oligodeoxynucleotide (ODN) binding to (NCC) membrane proteins. The binding activity of the ODNs were first confirmed by flow analysis of CpG binding to NCC. Figure 1 demonstrates that CpG binds approximately 36% of purified NCC whereas conjugate control binding was less than 5% (data not shown). To identify the membrane proteins that bound to the oligodeoxynucleotide, ligand (Southwestern) blots were done using NCC membrane lysates. NCC membrane preparations were analyzed by SDS-PAGE (12.5%) and blotted onto nitrocellulose. Membranes were probed with biotinylated GpC to identify the bacterial DNA binding proteins (Figure 2, lane 1). This ODN was previously shown [21] to compete with CpG

for binding to NCC. Four distinct molecular weight signals were identified: 14, 16, 18 and 29 kDa. Lane 2 is the conjugate control. The 18 kDa protein was excised and sent to Harvard Microchemistry laboratory for protein processing and fingerprinting analysis.

Sequence analysis of the DNA binding protein. One of the peptides identified by fingerprinting analysis had the following sequence: GASGSFKLNKK. Because this peptide had high similarity to trout H1, degenerate primers were designed based on this sequence. The cDNA product obtained following PCR amplification was sequenced. This product was used to synthesize nondegenerate primers to screen an NCC cDNA library and identify individual full-length clones of the gene coding for this novel protein. The cDNA product was translated and the complete amino acid (aa) sequence was submitted to NCBI (accession numbers AAQ99138 and AY324398) and is shown in Figure 3. The cDNA product was similar to mammalian histone 1X but was otherwise determined not to be identical to any previously described protein.

Characteristics of the DNA binding gene/protein. The gene encoding the ODN binding protein shown in Figure 3 has a typical polyadenylation signal and poly-A tail. Analysis of the open reading frame product predicted a protein of 22,064.63 Daltons composed of 203 amino acids. This protein has a pI of 10.75; it is composed of 58 strongly basic amino acids (K, R); 55 hydrophobic amino acids (A, I, L, F, W, V); and it has 50 polar amino acids (N, C, Q, S, T, Y). A database search for other proteins with similar and/or identical sequences to this novel protein revealed that it is similar to H1 histone family X proteins from zebra fish (zf) (192 aa), xenopus (217 aa), mouse (188 aa) and human (213 aa) (Figure 4). Amino acids 113-123 (Figure 4) were the source for the design of the original degenerate primers. This 10 aa sequence is identical to the original identified sequence by fingerprinting analysis except for a S115N substitution.

Table 1 compares the sequence obtained with H1X and H1 family members from both cold and warm blooded vertebrates.

Phylogenetic analysis of the novel catfish protein. Phylogenetic analysis confirmed the comparisons shown in Table 1. The catfish ODN binding protein clustered with zebrafish and xenopus histone 1X (Figure 5), showed a secondary similarity with mouse and human histone 1X, but was not related to any of the core histones (H2A, 2B, etc.).

Recombinant expression of the 22,064 Dalton membrane protein. The ODN binding protein DNA containing 6 x histidines (His-tag) was cloned into pET21b by standard techniques and the resulting plasmid was transformed into *E. coli* as described in Materials and Methods. In Figure 6A, purified recombinant ncamp-1 (containing the His-Tag) was identified with the INDIA His probe-HRP. In Figure 6B recombinant ncamp-1 from *E. coli* lysates was shown by Southwestern blotting to bind to GpC and CpG (lanes 2 and 5, respectively). dA20 was used as a negative control (lanes 7 and 8).

Specificity of a polyclonal anti-recombinant ncamp-1. A polyclonal anti-serum was generated against the recombinant ncamp-1. Figure 7A is the flow analysis of polyclonal anti-ncamp-1 binding to purified NCC. Approximately 53% of NCC constitutively express ncamp-1. The closed histogram is binding by rabbit pre-immune serum. The polyclonal antiserum also identified a 29 kD protein in a Western blot of NCC membrane lysates (Figure 7B).

Reciprocal competition binding. In order to confirm that the cloned catfish protein was indeed one of the NCC membrane proteins responsible for ODN binding, binding competition experiments between polyclonal antiserum and labeled GpC were performed with purified NCC. The flow cytometry histograms in Figure 8 show that preincubation of purified NCC with GpC prevents sequential binding of the polyclonal serum and vice versa.

Expression of a lysine based motif and comparison with histone-like proteins from other vertebrate species. Previous studies [31, 34, 37, 47-50] have identified lysine and proline rich antimicrobial peptides (AMP) from various teleost species. The similarity between the N-terminus of ncamp-1 and three different antimicrobial H1 peptides is shown in Figure 9. The most striking feature of each sequence is the predominance of lysine, alanine and proline as contiguous sequences or as xAKx, xKAKxx or xKKAx motifs. These motifs are frequently boxed by lysine (e.g. KxxxK). An algorithm would suggest that these lysine boxed motifs (LBM) may be responsible for ligand binding and/or antimicrobial functions. Similar comparisons of the LBM in the C-terminal 60 aa of ncamp-1 with core and linker histone peptides confirmed this relationship and emphasized that although there are no sequence identities, that the similarities in expression of the LBM may impose important biological consequences.

Antimicrobial effects of recombinant ncamp-1. Because antimicrobial activities of low molecular weight histone-like peptides have previously been described (33-38), studies were next carried out to evaluate the bactericidal activity of ncamp-1. Figure 10 demonstrates that both *E. coli* and *S. iniae* were killed by the full length recombinant ncamp-1 (a similar killing curve was generated in the presence of *Micrococcus luteus*; data not shown). In addition, truncated forms of ncamp-1 were also tested and are shown in Figure 10A and Table 2. Two different truncated recombinants (ncamp-d1 and ncamp-d2) killed *E. coli* at MIC₅₀ concentrations of 0.63 uM and 1.0 uM respectively.

DISCUSSION

Naturally occurring antimicrobial proteins and peptides (AMP) have been identified from a wide diversity of plant, invertebrate and vertebrate species [40-44]. These AMP have been

classified based on both chemical and conformational properties and they can be differentiated based on whether the active form is a peptide (i.e. 17-35 aa in length) or a protein (>50 aa). An additional distinguishing property of AMP is their cationic nature with little to no amino acid sequence identity across all the members of this very large group. For example, cecropins, magainins and defensins from silk moth, *Xenopus* and mammals (respectively) are all low mw AMP. Although they share lysine rich regions and are inducible, they have no sequence homology. The functional characteristic of this large group of AMP is based on their common ability to kill bacteria and (in some cases) eukaryotic cells.

The amino acid content of these AMPs provides clues regarding the common chemical and physical features that may be responsible for their bactericidal effects. An example is the recently described AMP Cupiennin-1 [45]. This 35 aa basic peptide has 8 lysine residues, is present in the venom of *Cupiennius salei* (a hunting spider found in Central America), is amphipathic and has bactericidal activities against Gram negative and Gram positive bacteria. This peptide may be similar to other AMPs (e.g. magainins; 46) regarding the mechanism of binding to bacterial cells. It was predicted to fold into an amphipathic alpha-helix when it inserts into the bacterial cell membrane. Differential sensitivities of eukaryotic versus prokaryotic cells are thought to be based on the low cholesterol content and relatively high negative charge density of bacterial cell walls compared to eukaryotic cells (46).

Another type of AMP is not naturally occurring but is generated *in vitro* by proteolytic digestion or acid hydrolysis of some precursor, larger mw molecule. These AMP are relevant innate immune response effector substances. One interesting class has been studied in species ranging from teleosts to humans and is composed of histone like proteins. The traditional cellular location of histone proteins (H1) is in the nucleus associated with chromatin fibers either

in the form of linker histone 1 or core histones (H2a, H2b, H3 and H4) that form nucleosomes. However, studies performed in higher vertebrates have shown that many cells of the immune system express cytoplasmic and membrane forms of these proteins [47-49]. Previous reports by others have shown that a relatively wide variety of human cells express histone like membrane proteins (HLMP) [22-31]. The function(s) of HLMP cationic proteins have generally not been ascribed to ligand or receptor activities except for thyroglobulin binding by an H1 receptor on mouse macrophages [32] and DNA binding by a 28 kDa protein on “normal” human lymphocytes [50]. Comparatively, two major categories of DNA binding proteins exist on the membranes of antigen presenting cells from vertebrates. These are Toll-like receptor 9 [51-54] and Scavenger receptors [55-56]. These receptors bind to unmethylated CpG oligodeoxynucleotides and polyguanosine respectively. Such receptor-ligand interactions generally function to initiate signaling during innate immune responses.

Toll-like receptor proteins have not yet been identified on teleost cells although a trout homologue has been obtained from an EST library of differentially expressed liver genes [57]. As this sequence corresponds to the leucine rich repeat (LRR) domain, its role as a bDNA binding protein is uncertain. Similarly, two additional EST's from zebrafish (Accession numbers: BF158452 and BG304206) have identified fragments of Toll-like genes, but their functions also remain unknown. Complete sequencing and functional characterization of these molecules and other PRR will provide invaluable insight into the evolution of these receptors and their role in pathogen resistance.

We have previously shown [21, 58] that teleost NCC bind oligodeoxynucleotides. This binding is receptor mediated. Competition binding experiments demonstrated that CpG and GpC bound to the same receptor on NCC. The fact that scavenger receptors are not known to bind

either CpG or GpC [59] and that membrane expression of TLR9 protein has not previously been described for fish cells [60-61] [although a full length TLR-like sequence has been reported for a goldfish macrophage cell line; 62], we hypothesized that NCC might express a novel class of binding protein(s) responsible for this activity. In the present study we identified a new DNA binding receptor with molecular similarities (less than 50% at the aa level) to histone 1X (Figure 2). A search of the zebrafish sequences in the NCBI database revealed a similar protein, but with “unknown” functions (accession number AAH47192). The zebrafish protein has 51% similarity to the novel catfish protein (Table 1). Expression of the catfish DNA binding protein in other hematopoietic cell lines and tissues was verified by searching a channel catfish EST database and this gene expression was reported in the NK like cell line MLC-52-1 (accession numbers CB937576 and CB937396), brain (accession number BM495146) and in the anterior kidney (accession number BE469379).

Data from our laboratory has shown that, similar to histones, the ncamp-1 gene does not have introns (data not shown). The phylogenetic analysis of the ncamp-1 with other histone-like proteins indicated that this protein could be a separate evolutionary branch from the histone-like protein family (Figure 5). Although ncamp-1 appeared related to the histone family, more closely to H1 histone family X members, this relationship was based on similarities in the conserved central domains in these proteins. The exact physiological functions of histone H1 family X members are still not understood. Present observations will contribute towards various predicted functions for histone family members other than nuclear assembly.

Ncamp-1 was next examined for unique domain configurations, amino acid repeats or presence of conserved motifs. The periodic expression of multiple and sequential lysine residues flanked by alanine (Figure 3) throughout the molecule suggested similarities to histone-like

proteins previously described by others [34, 37, 38, 47-53]. These HLP have been described to have antimicrobial activity. Comparisons of ncamp-1 and HLP (Figure 4) revealed that the multiple lysines (when expressed) were arranged in boxes characterized by: KxxxK, KKxxK and KxxKK) with an apparent “preference” for flanking alanine and proline amino acids. We referred to these repeats as lysine box motifs (LBMs). Because of the similar relationship of (naturally occurring) ncamp-1 with histone 1X proteins from other vertebrates, we next determined whether the LBMs were conserved in any phylogenetic relationship with processed peptides from other species. In Figure 7, several low mw peptides that have been previously shown to have antimicrobial activity are compared with N- and C-terminal domains of ncamp-1 for expression of LBMs. HLP from mouse, human and three different species of fish all express similar LBMs. Comparisons of the LBM from ncamp-1 with naturally occurring antimicrobial peptides (e.g. Bacteriocin/P80214; Cecropin A/M63845; Cupiennin 1a/P82358; Buforin I/x011064; and Magainin II/A29771) revealed a similar pattern of LBM expression analogous to that seen for ncamp-1. These comparisons also demonstrated that there were essentially no sequence identities between peptides and OBP. Examples of other AMP not shown but that also expressed multiple LBMs are adenoregulin from the leaf frog (*Phyllomedusa bicolor*: containin 3 LBMs; accession #P31107) and brevinin-2E (containing 2 LBMs; accession # S33730) from the European frog (*Rana esculenta*).

A recombinant form of ncamp-1 was expressed in *E. coli* and tested for binding to ODNs. The purified histidine-tagged recombinant protein had an apparent mw of 29-30 kDa identified by Western blot examination using His-Probe HRP (Figure 6). The apparent mw discrepancy is produced by the abundant lysine residue content of this protein. This phenomenon has been

previously reported for other histone-like AMP (63) that have a lower computed molecular weight compared to their (experimental) electrophoretic mobilities.

A similar abundance of molecular and immunological information regarding the effects of ODN binding to mammalian cells is not available for teleosts. In these cold blooded vertebrates, histone proteins and peptides with antimicrobial activities have been isolated from salmon blood, liver, intestine and mucus [64]. Catfish skin, epithelial cells and mucus contain H2A-like (Parasin-I) and H2B-like molecules [38]. These studies demonstrated that histone release from cells required tissue injury and thus, membrane expression of histone-like proteins was not determined. CpG-induced activation of rainbow trout macrophages was determined by induction of IL-1 β and IFN-like cytokines [65]. Similarly, CpG activated leukocytes from Atlantic salmon had increased interferon production (66). We recently demonstrated [58] that NCC from teleosts can be directly activated by CpG ODNs to induce cytolytic activity against tumor target cells. An ODN hierarchy for activation of cytotoxicity was shown: (a) ODNs composed of GpC motifs had the highest activity when followed by the sequence –AACGTT-; (b) similar to mammals, the hexamer palindrome sequence of 5'-pu-pu-CpG-py-py-3' was also stimulatory in teleosts where 5' purines are preferably expressed as GpT and 3' pyrimidines are TpT (21); and DNA binding proteins on NCC also recognized polyguanosine oligodeoxynucleotides.

Our study supports the notion that teleosts are a relevant model to examine innate immunity in both warm and cold-blooded vertebrates. Ncamp-1 has been identified on catfish and tilapia NCC as well as on mouse [58] and human cells (data not shown). This receptor may participate as a positive immunoregulator during bacterial infections and as such provide an important and necessary effector function in the survival of vertebrate species.

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Figure Legends

Figure 1. ODNs bind to NCC and NCC membrane proteins. A. Rhodamine-labelled ODN (CpG; 40 ug) was added to purified NCC and analyzed by flow cytometry. Thirty six percent of NCC were positive for constitutive ODN binding.

Figure 2. Southwestern blot analysis of membrane lysate prepared from NCC. Blots were probed with biotinylated ODN (GpC) and Extravidin-HRP (Lane 1) or Extravidin-HRP only (Lane 2). Four proteins (14, 16, 18, and 29 kDa) were identified. The 18 kDa protein was excised from Coomassie stained gels and submitted for trypsin digestion/MS analysis. Lane 1 shows binding by biotinylated-GpC. Four distinct proteins (14, 16, 18 and 29 kDa) bind to GpC. Lane 2 is the conjugate control. The 18 kDa protein (arrow) was submitted for amino acid analysis.

Figure 3. Compiled full-length catfish ncamp-1 cDNA sequence. Lysine residues are represented in bold letters. Polyadenylation site is highlighted and poly A tail is underlined. Start and stop codons also are represented in bold letters.

Figure 4. Comparisons of the deduced amino acid sequence of catfish DNA binding protein with histone H1 proteins from different species (zebrafish, Xenopus, mouse and human).

Boxed area was also identified from primary sequencing and was the source for design of original degenerate primers. Multiple sequence alignment comparisons were made using CLUSTAL W.

Figure 5. Phylogenetic analysis of catfish ncamp-1: Phylogram showing relationships of catfish ncamp-1 to other histone-like proteins. The tree was derived by parsimony analysis, with Mega version 2. Numbers shown above the branches are bootstrap values based upon 1000 replicates for parsimony. A separate analysis using maximum likelihood and neighbor joining

methods produced a tree with similar topology. The tree was rooted on a sub-tree containing histone H2 and similar proteins.

Figure 6. The catfish histone H1X-like ODN binding protein was expressed in *E. coli* as a 6x His-tagged recombinant. Panel A- The purified recombinant ncamp-1 migrated at approximately 31 kDA when probed with His-Probe HRP. Panel B- Southwestern blot analysis demonstrates binding to the purified (P) recombinant ncamp-1 by both biotinylated GpC and CpG, minimally by biotinylated polyadenine (dA20) but not by Extravidin-HRP (Control). L: whole cell *E. coli* lysate prior to purification; and V: NiNTA column eluate from vector only transformed lysate treated the same as L and P.

Figure 7. Polyclonal anti-NCAMP binds NCC and recognizes a 29 Da protein in NCC membrane lysates. Panel A. Purified NCC were incubated with anti-ncamp-1 IgG (open histogram) or control rabbit IgG (shaded histogram) and anti-rabbit IgG FITC. Cells within the cursor were considered positive and were 53% of the total. Panel B. Blots of NCC membrane lysates were probed with anti-ncamp-1 IgG (Lane 1), control rabbit IgG (Lane 2) or anti-rabbit IgG HRP only.

Figure 8. Anti-ncamp-1 and oligodeoxynucleotide bind to the same protein on NCC.

Catfish anterior kidney cells were examined by competition binding experiments. Panel (A) demonstrates that GpC-Biotin, but not dA20-Biotin inhibited binding by polyclonal anti-ncamp-1. Panel (B) shows that GpC competitively inhibited binding by anti-ncamp-1 to NCC. In panel A: the positive control anti-ncamp-1 binds to NCC; addition of dA20 prior to anti-ncamp-1 does not prevent antibody binding; and GpC-Biotin prevents anti-ncamp-1 binding. In the left panel (B), reciprocal binding competition between GpC and anti-ncamp-1 demonstrates the same binding specificities. In the right panels (B), the appropriate controls are shown.

Figure 9. Identification of lysine boxed motifs in the OBP and comparisons with other antimicrobial proteins for motif similarities.

Figure 10. Antimicrobial assay. Effects of full length and truncated recombinant ncamp-1 on *E. coli* and *Streptococcus iniae* viability. The bacteria were prepared and recombinants were expressed as described in the Materials and Methods. The micrograms of recombinant shown represent that amount in 100 ul treatment volume and the dotted line indicates initial number of bacteria added to the treatment wells.

Figure 1.

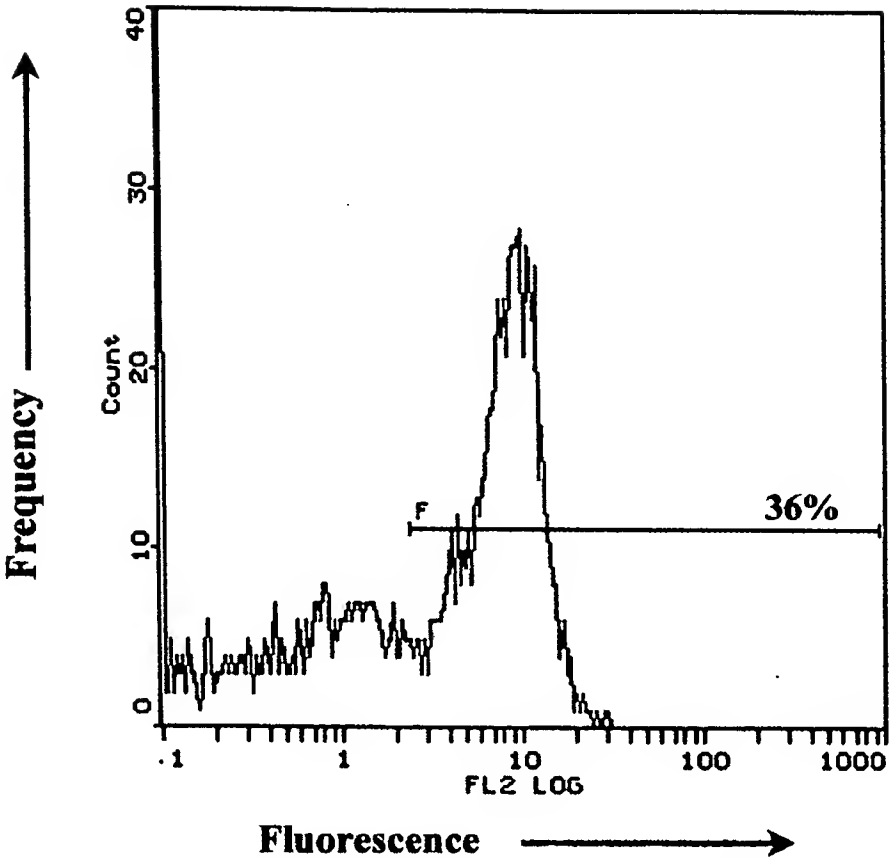
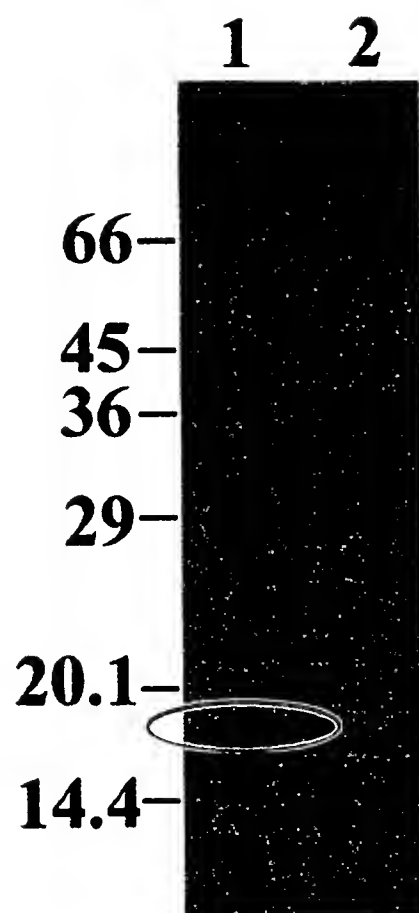


Figure 2.



1 CGGCACGAGGGTTCAATAGCATCTCAAGGCGCTTCAGAACTTAAAGTTGA 16
M S A Q A E E T A P E A A A P V
51 ACC**ATG**TCTGCTCAGGCTGAGGAACTGCACCAGAAGCAGCAGCACCAGT 32
Q P S Q P A A **K K K** G P A S **K A**
101 ACAACCATCACAAACCAGCGGCCAAAAAGAAGGGACCCGCCAGTAAAGCAA 49
K P A S A E K K N K K K K G K G P
151 AGCCTGCCTCTGCAGAAAAAAGAACAAAAAGAAGAAAGGGAAAGGGCCC 66
G **K Y S Q L V I N A I Q T L G E R**
201 GGAAAGTACAGCCAGCTGGTGATCAATGCTATCCAAACGCTGGGAGAGAG 82
N G S S L F **K I Y N E A K K V N**
251 AAACGGCTCGTCTCTTTTTTAAGATCTACAACGAGGCGAAGAAAGTGAAGT 99
W F D Q Q H G R V Y L R Y S I R A
301 GGTTTGACCAGCAGCACGGGCGCGTGACCTCCGCTACTCCATCCGCGCG 116
L L Q N D T L V Q V **K G L G A N G**
351 CTGCTGCAGAACGACACGCTCGTGAGGTGAAGGGTCTGGGCGCCAACGG 132
S F **K L N K K K F I P R T K K S**
401 CTCCTTCAAGCTCAACAAAAAGAAGTTTCATCCCCAGAACCAAGAAGAGCT 149
S V **K P R K T A K P T K K P A K K**
451 CTGTAAAGCCGAGAAAGACTGCGAAACCGACCAAAAAGCCAGCCAAAAAA 166
A A **K K K K R V S G V K K A T P P**
501 GCAGCGAAGAAGAAGAAAAGGGTCAGCGGCGTGAAGAAGGCGACTCCCCC 182
P E **K T S K P K K A D K S P A V**
551 CCCAGAGAAAACCTCCAAACCAAGAAAGCGGATAAAAGTCCAGCCGTCT 199
S A **K K A S K P K K A K Q T K K T**
601 CTGCCAAGAAGGCGAGCAAGCCCAAGAAAGCTAAACAGACAAAAAAGACT 203
A **K K T ***
651 GCTAAGAAGACTT**AAAA**ACGTTTATATTCTGCATGCTTTGTGCATTAAAGCA
701 TTGCACTGCGGGTAAACTGCACGCTTTCTGATCGCAGTTCATTAAAGTAGG
751 ATATGCACAGTGTTTAACCAAGTGTGCAAGTCACTCTGGTCTCAATGTTT
801 TACTGATGTAACCACATGTAAATAACTGTACAAAGAAGGAAACAATCACT
851 TTTGTAAACGTCTGCTTTGTTATTATTTCTTTTCTACTAGTTAGCTAAAT
901 AACTGCTTATGGCTTCTTTTAA**ATATA**ATGATAAAAG**AAAAAAAAAAAA**
951 **AAAAAA**

Figure 3.

Figure 4.

```

Catfish NCAMP-1      : MSAQAEETAPAAAPVQPSQP-----AAKKKGPAKPKPASAEEKNNKKKGKGQINAQTIN : 68
Danio H1X-like-AAH47192 : ----MPAVVESAPAPAPAP-----AEKKAKPAVASPAKK---KKKSKGKTDA RTIN : 59
Xenopus H1X-AAH41758   : ----MALELENLHSTEEDEEEEEEGDEMRSRSTRNKGGASSSGNKKKKK--KNQKVDTIRKIN : 73
Mus H1X XP_144949      : ----MSVEL EALPPTSADG-----TARKTAKAGGSAPTQPKRRKN-RKNQKVETIRKIN : 63
human H1X-BAA11018     : ----MSVEL EALPVTTAEG-----MAKKVTKAGGSALSPSKKRKNKKNQKVETIRRIN : 64

Catfish NCAMP-1      : FNNCHVMAQFIPTKKSSVKPRKTAKPTKKPAK : 148
Danio H1X-like-AAH47192 : FNNCHVMAQLEKKPKK-AASKKATKKTEKPTSK : 138
Xenopus H1X-AAH41758   : FNNCHVMAQLEGLPYDKKPPPAKPSSSSSNKK : 153
Mus H1X XP_144949      : FNNCHVMAQLEGGAEERR-GASAASSPAKAR-- : 140
human H1X-BAA11018     : FNNCHVMAQLEGGGERRGAPAAATAPAPTAHKA : 144

Catfish NCAMP-1      : KAAK----KKKRVSGVKATPPPEITSIPK-----KADKSPVSAKASPKAKQTKTAKIT- : 203
Danio H1X-like-AAH47192 : KAVT----KKVSAKKSAKSPVKKTPTKT-----SVKKATKPKTASKPAKAAKKTKS-- : 192
Xenopus H1X-AAH41758   : QQQ----GPSSSPSKSHKAKPKAAE EKPKTSSAKAKSPKKSAG--KMKGA PSVRAPKSKA : 217
Mus H1X XP_144949      : -----TAAADRTPAIPQ-PERAH S-----KKAASASAKVKAAAPSVPVPKGK- : 188
human H1X-BAA11018     : KKAAPGAAGSRRADKKPAIGQKPEQSHKAGAGAKDKGGKAKKTAAGGKVKAAAPSVPVPKGK- : 213

```

Figure 5.

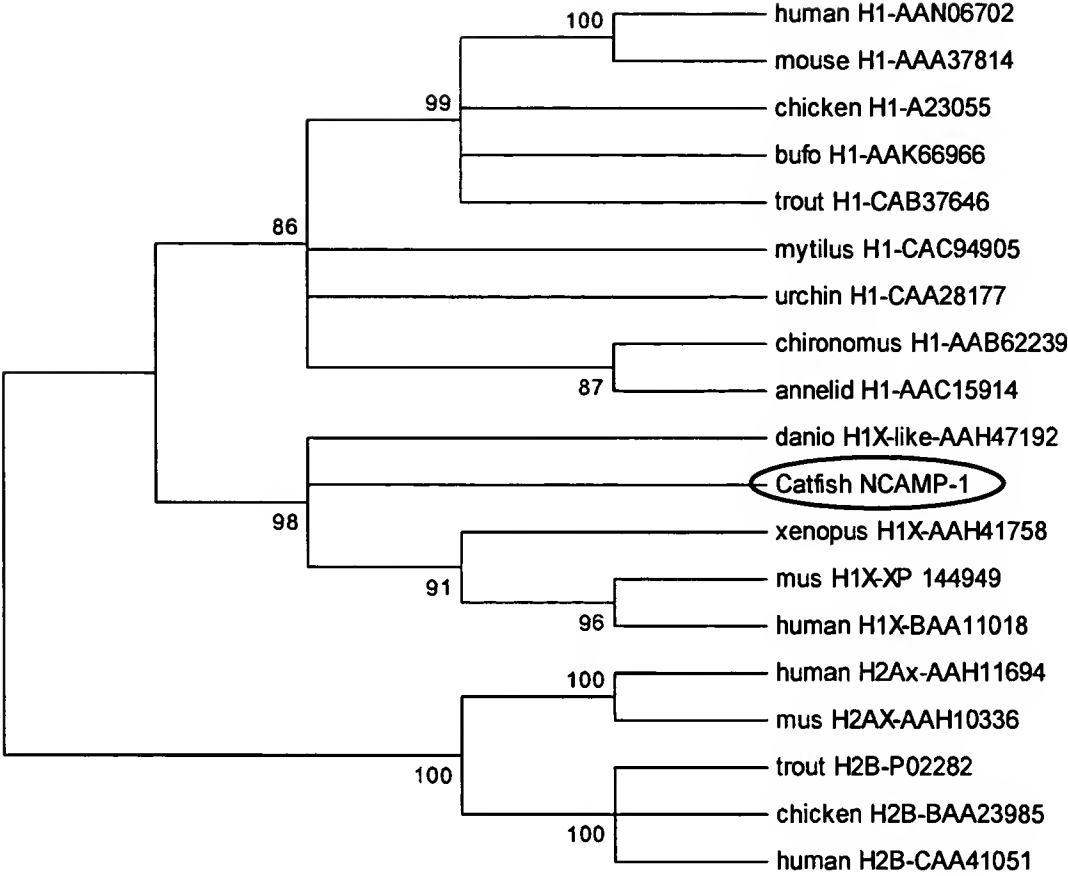


Figure 6.

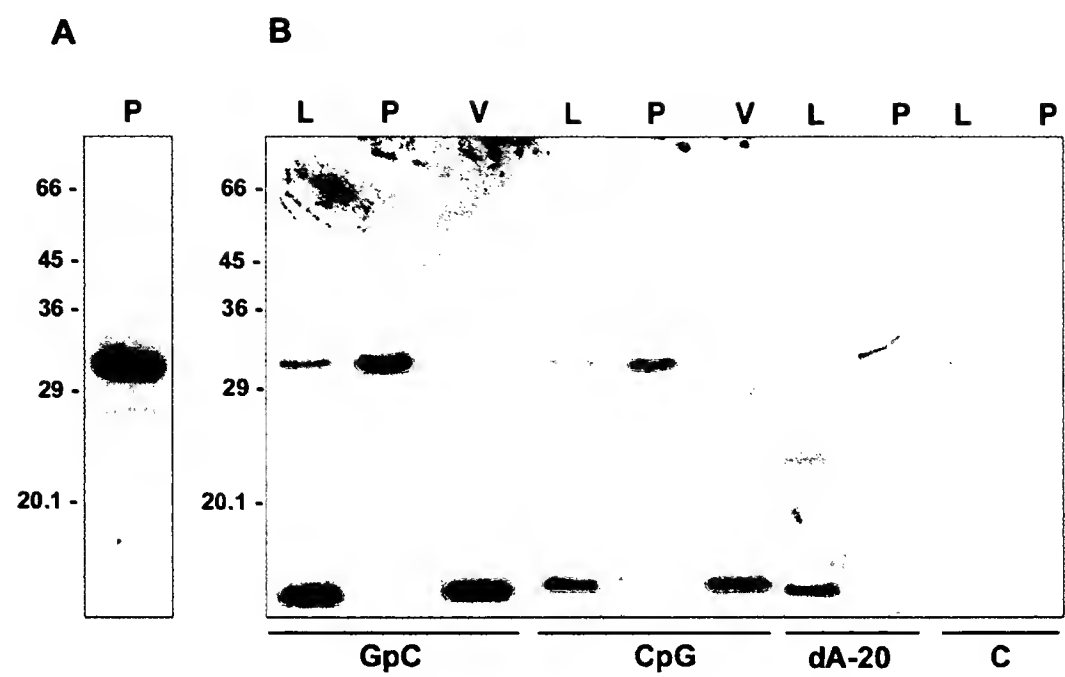
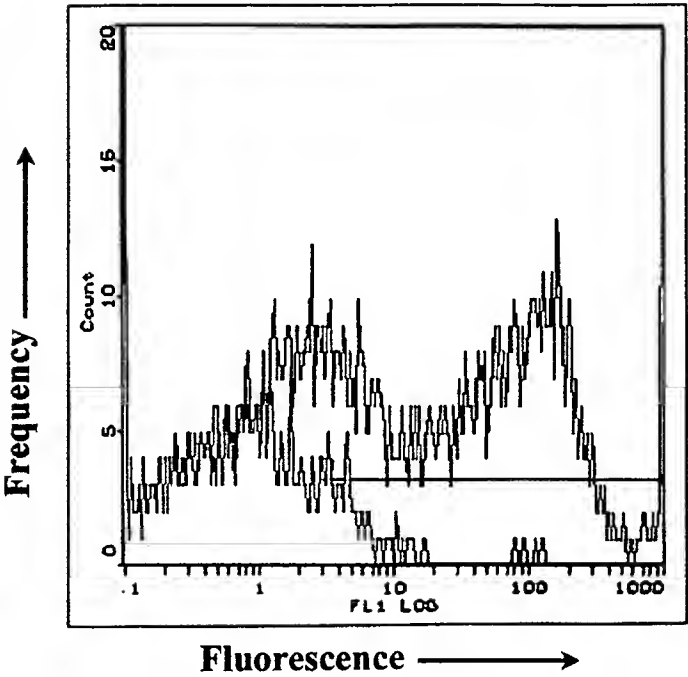
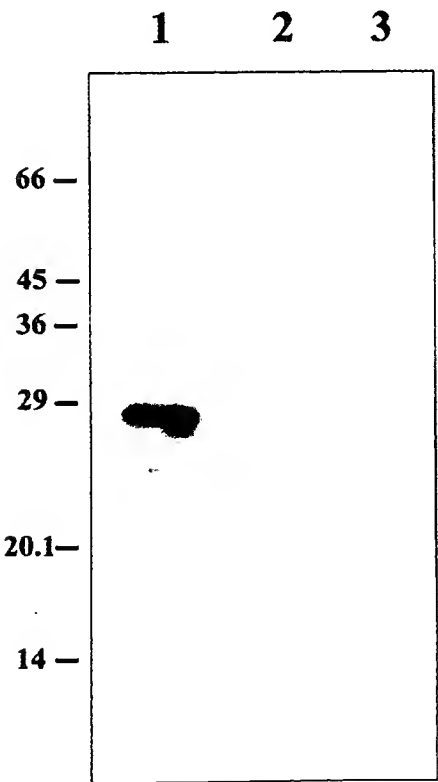


Figure 7.

A



B



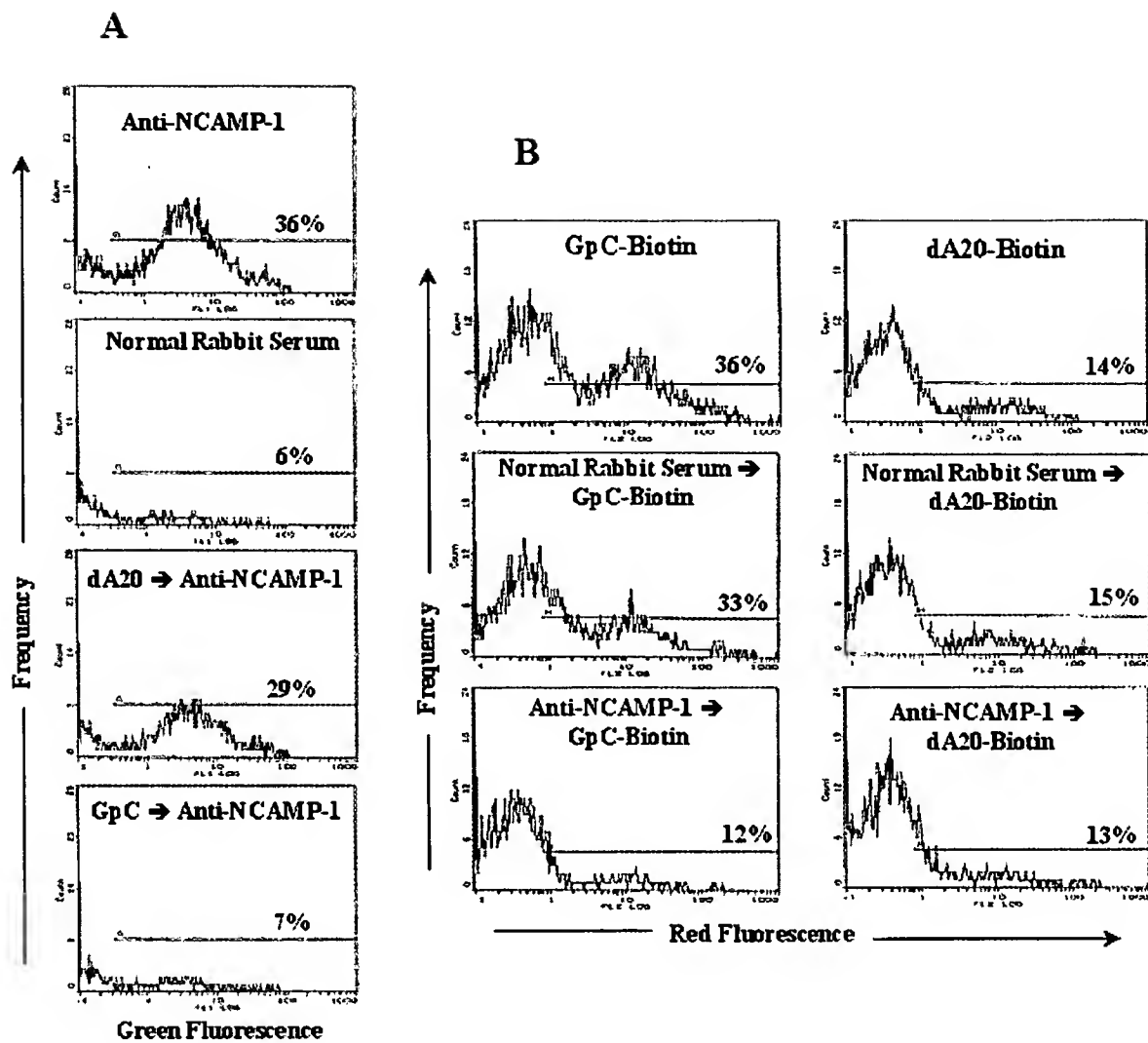


Figure 8.

Figure 9.

N-Terminal residues:

NCAMP-1 10 PEAAAPVQPSQPAAKKKGPASAKAPSAEKKNKAKGKGPG 50

H1-Mus ¹ (68) -----SETAPAEKPAPAKAE--

H1-Human (29) ---KLNKKAASGEAKPKAKAKSPKKAKA--

H1-Trout (36) -KAVAAKKSEPKKAKKPAT--

C-Terminal residues :

NCAMP-1
139 -TAKPTKKPAKKAACKKKRVSGVKKATPPPEKTSKPKKADKSPAVSAKKASKPKKAKQT 196

H2A CF (37) --KGRGKQGGKVRAKAKTRSS--

H2B Trout^(A) -----PDPAKTAPKKGSKKAVTKXA--

H2B Bass1 (34) -----PEPAKSAPKKGSKKAVT-

H2B Bass2^(B) (34) -----PDPAKPTAPKKGSKKAVTKTAG

H1-Trout^(C) -----AEVAPAPAAAAPAKAPKKKA

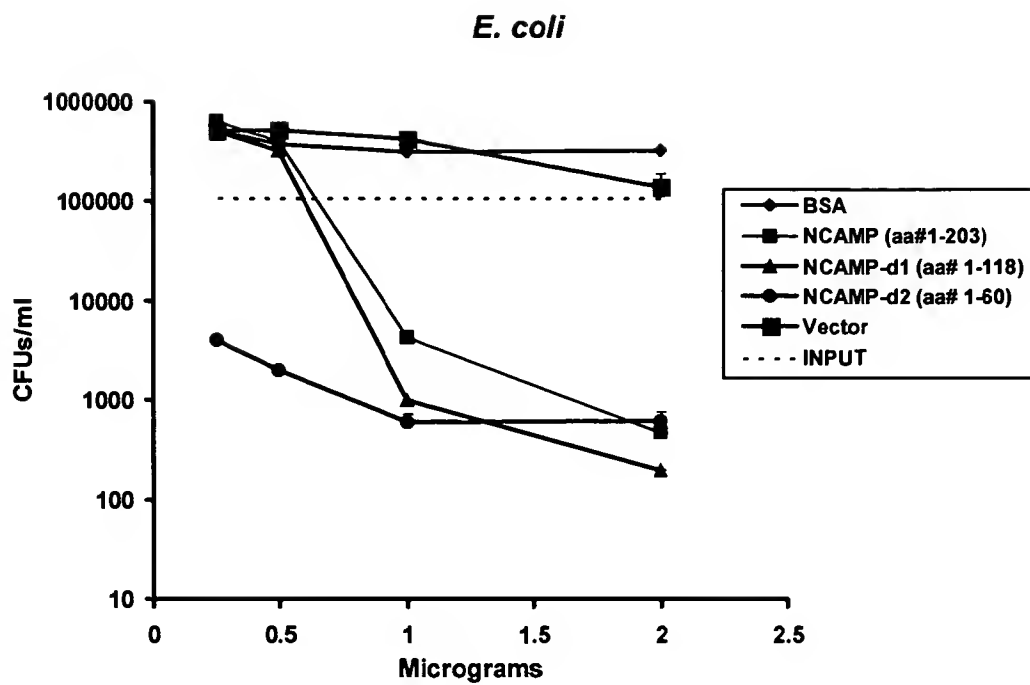
H1-Trout (34,35) ---AEVAPAPAAAAPAKAPKKKAAAKPKK-----

References

- ^(A) Robinette, D., Wada, S., Arroll, T., Levy, M. G., Miller, W., and Noga, E. J. (1998). Cell Mol Life Sci, 54:467-475.
- ^(B) Kootstra, A. and Bailey, G. (1978). Biochem. 17:2504-2510.
- ^(C) Macleod, A. R., Wong, N.C.W. and Dixon, G. H. (1977). Eur. J. Biochem., 78:281-291.

Figure 10.

A



B

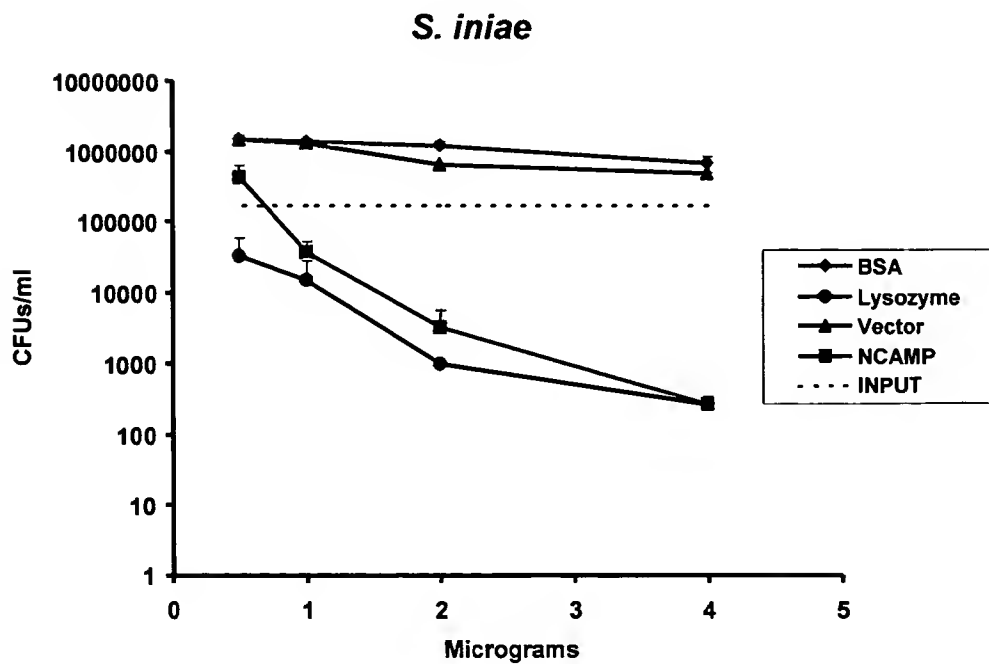


Table 1. Amino acid identity of catfish DNA binding protein to other histone-like

proteins. HIXHUM: H1 histone family member X from human (Accession # BAA11018), H1XMUS: H1 histone family member X from mouse (Accession # XP_144949), HIX-Xen: H1 histone family member X from *Xenopus levis* (Accession # AAH41758), H1TRT: Histone H1 from trout (Accession # CAB37646), H1HUM: Histone H1 from human (Accession # P10412).

		<u>H1X</u>				<u>H1</u>	
		Danio	Human	Mouse	Xenopus	Trout	Human
DBP		51.2	42.4	43.9	42.9	30.3	33.1
H1X:	Danio	---	44.1	41.3	42.9	34.7	33.9
	Human	---	---	68.5	53.6	28.6	26.7
	Mouse	---	---	---	50	26.9	25.1
	Xenopus	---	---	---	---	26.7	26.6
	Trout	---	---	---	---	---	61.9

Table 2. Minimal inhibitory concentrations for 50% bactericidal activity for full length and truncated ncamp-1.

Species	Protein/Peptide	¹ MIC ₅₀
<i>E. coli</i>	ncamp-1, aa 1-203	0.43 uM
	ncamp-d1, aa 1-118	0.63 uM
	ncamp-d2, aa 1-60	1.0 uM
<i>S. iniae</i>	ncamp-1, aa 1-203	0.86 uM

¹MIC for 50% bactericidal activity.

TELEOST ANTIMICROBIAL AND CYTOTOXIC POLYPEPTIDES

Anti-bacterial Activity of Soluble Recombinant NCAMP-1

Soluble recombinant NCAMP-1 has been shown in an anti-bacterial assay to kill both a gram negative (*E. coli*) and gram positive (*Micrococcus luteus*) bacteria. The killing capacity of NCAMP-1 against clinical isolates of a fish pathogen, *Streptococcus iniae* has been studied. Growth and anti-bacterial assay conditions have been established for 2 of these isolates. Results indicate strain dependent sensitivity to the antibacterial activity of NCAMP-1. (ie. Isolate KFP164 is susceptible while isolate DAN 14 is completely resistant). Preliminary characterization of 10 additional clinical isolates and a reference strain (ATCC) similarly reflect strain dependent sensitivity to NCAMP-1 activity.

NCAMP-1 Truncation Constructs.

Rationale . Analysis of hydrophobicity plots of NCAMP-1 together with known HI domain structures indicate a tripartite molecule with a central hydrophilic helical domain flanked by charged hydrophobic domains. We determined which of these domains are necessary (sufficient) for antimicrobial activity and DNA binding activities.

Inspection of NCAMP-1 sequence indicated the following constructions for testing:

- NCAMP-1 — Full length, amino acids 1-203 (shown to have anti-microbial activity as indicated above)
- NCAMP-d(deletion)1 —Amino acids 1-118; (deletion of the C-terminal charged, hydrophobic domain leaving the N-terminal charged hydrophobic and central hydrophilic domains).
- NCAMP-d(deletion)2 —Amino acids 1-60 (deletion of both the central and C-terminal domains leaving only the N-terminal charged hydrophobic domain).

PCR was used to generate restriction sites for insertion of constructs into the BamHI-XhoI site of pET 21b (Novagen). Ligation into this site results in expression plasmids with a C-terminal 6xhis tag. Plasmids were initially transformed into *E. coli* strain DH5a for stock plasmid production before transforming into the WIG-inducible *E. coli* expression strain BL21(DE3) pLysS (Novagen). Cultures were induced after growth to OD600 0.6-1 and cells were harvested 3 hours later. Cell lysates were prepared by treatment with lysozyme, Triton X-100, DNaseI and RNaseA. Recombinant protein was recovered with nickel chelating resin (NINTA agarose, Qiagen) according to the manufacturers protocol.

Testing of these constructs indicates that the d2 construct, but not the d1 is capable of anti-bacterial activity against *E. coli*. Further, the dose response curve of d2 activity appears similar to that of full length NCAMP-1. In contrast, DNA binding activity (as determined by ODN blotting experiments) is completely abolished in both d1 and d2 constructs indicating the C-terminal domain is necessary for DNA binding activity.

Nonspecific Cytotoxic Cells (NCC) express Anti-Microbial Protein (AMP) that recognizes bacterial DNA and degrades it. This is a membrane protein. It kills Gram positive and Gram negative bacteria.

Oligonucleotide (ODN) binding to NCC membrane proteins—

CpG binds 36% of purified NCC by flow analysis

Ligand (Southwestern) Blots done using NCC membrane lysates—to identify bacterial DNA binding proteins. Four Found: 14, 16, 18, 29 kDa

Amino Acid sequence analysis of 18 kDa protein

Recombinant DNA expressed—22064 Da membrane protein

Lysine (K) rich protein with sequences such as KxxxK which may be responsible for ligand binding or antimicrobial function.

Soluble recombinant NCAMP-1: strain-dependent sensitivity to NCAMP-1 activity.

Hydrophobicity plots indicate three domains in NCAMP-1. There is a central hydrophilic helical domain with charged hydrophobic domain at either end.

Truncation constructs indicate N-terminal domain alone but not two N-terminal domains together is capable of antibacterial activity against *E. coli*. Dose-Response curve is similar to that of the native protein.

The C-terminal domain is necessary for DNA binding activity.

Claims:

1. All compounds, compositions, methods and use of the compounds, compositions and methods as otherwise described herein.
2. Antimicrobial and/or cytotoxic polypeptides and peptide regions of polypeptides as otherwise described in the present application.
3. Recombinant DNA expressing polypeptides.
4. Vectors expressing disclosed polypeptides.
5. Methods of producing polypeptides.
6. Binding of ODN to membrane protein.
7. Purified ncamp-1, cDNA containing DNA sequence for ncamp-1 and vectors comprising the DNA sequence.
8. Methods of inhibiting bacteria and other microbes using cytotoxic polypeptides as described herein.
9. A method of treating an animal, including a human, for a microbial infection comprising administering an effective amount of one or more of the polypeptides according to the present invention to said patient to inhibit said microbial infection.
10. The method according to claim 9 wherein said infection is a bacterial infection and said animal is a human.